Blood supply to human malignant melanoma xenografts in the mouse dorsal window chamber

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ABSTRACT

The effectiveness of non-surgical treatments is depending on the oxygenation status of the tumor. The oxygenation status is determined in part by whether the bloodsupply is arterial or venous in origin, if the blood is distributed homogenously troughout the tumornetwork, and if there is fluctuations in the bloodstream. We have looked at some of these parameters for the malignant melanoma xenographt, using the mouse dorsal window chamber and a fluorecent cell line. We have also developed a techinque where we follow a bolus of contrast as it moves through the tumor vessel network. This first pass imaging technique has not been combined with vital microscopy before.

We found that the first bloodsupply to the tumor was arterial in origin, and it remained so troughout the entire test periode. The blood reached some parts of the tumour with a time lag compared to other parts of the tumor. We also found that the blood passes through the tumor tissue significantly faster than through the normal muscle tissue.

In short, the tumor is supplied by blood with high oxyhaemoglobin saturation that moves quickly through it; this speaks in favor of the tumor being well oxygenated. In contrast, some areas of the tumor recives blood that has passed through a longer stretch of vessel than other parts, and these parts will consecuently be more at risk for hypoxia than other parts if the oxyhemoglobin saturation or blood flow is reduced. Hypoxic regions in a tumor select for more malignant and metastaic cells, and diminishes the effect of non-surgical treatment.

INTRODUCTION

Tumor cells require oxygen and nutrients, and must be located no more than 100-200µm from a vessel to get a sufficient supply, as this is the limit for oxygen diffusion (Charmeliet and Jain 2000). The PO_2 drops exponentially with increasing distance to the nearest vessel, and when the distance exceeds 200µm, the PO_2 dropes to hypoxic values. (Filho *et al*1994)
Whithout blood vessels, tumours cannot grow beyond a critical size or metastasize to another organ.(Charmeliet and Jain 2000) Both hypoxia and acidosis increase the activity of some angiogenic promoting factors, (Fukumura *et al*2001) leading to angiogenesis.
Tumour vessels are structurally and functionally abnormal. In contrast to normal vessels, tumour vasculature is highly disorganized; vessels are tortuous and dilated, with uneaven diameter, excessive branching and shunts (Charmeliet and Jain 2000). This leads to some areas of the tumor beeing well vascularized, whereas others have little or no blood supply. (Jain 1994).
If there is a deficient bloodsupply to parts of the tumor, it may not be possible to deliver anti-cancer drugs to all regions of a tumour in effective quantities. In addition, hypoxic conditions select for cancer cells that are more malignant and metastatic. (Charmeliet and Jain 2000)
Hypoxic conditions can have an antagonistic result when coupled with radiation therapy (Murata *et al*1997) and chemotherapy (Ma *et al*2001). If the tumor vasculature is instead

treated so that it normalizes in respect to diameter, density and permeability, it can have a synergistic effect when coupled with radiation or chemotherapy (Jain 2002).
It is therefore important to study the develoment of the vasculature of different tumors, and how it responds to antiangiogenic therapies, so that the treatment can be optimalised.
The purpose of this study is to assess whether the initial bloodsupply to the tumor is arterial or venous in origin, as this will influence the oxyhaemogbin saturation. We also aim to see if this changes as the tumor grows. For this purpose we use the dorsal skinfold chamber, witch is suited fore repetetive observations of the spatial and temporal relations of neoangiogenesis, and the functional state of the newly developed microvasculature (Vaikoczy *et al* 1998). We want to find out if there are regions in the tumor in witch the blood has passed through the tumor tissue for å longer time than what is normal for capillaries in muscle tissue.The probability of observing fluctuations in blood perfusion in these areas will be increased, as there is a longer network of tumor vasculature upstream. Fluctuations here will more easily cause hypoxia. To study this we use first pass imaging, a technique that monitores the bloodflow through the tumor directley by following a boulus of i.v. injected contrast.
The knowledge from this study can then form the basis for further studies involving antiangiogenic substances and other non-surgical treatments.

MATERIALS AND METHODS

Spheroids

Green Fluorecence protein (GFP) -transficted malignant melanoma cells from the A07 line, obtained from The Norwegian Radium Hospital, department of Biophysics, were used. Non-transfictated cells of the A07 strand, was rinsed with PBS, and incubated with a mixture of plasmide DNA, transfection medium (OPTIMEM1, Catalogue nr 31985-047, Gibco BRL) and catatonic liposomes for 6 hours at 37ºC. The transfection mix was then exhanged for 10 ml cell medium, and the incubation was continued for 20 hours at 37ºC. The cells were thus transfictated with the GFP gene. The cells with the strongest and most homogenous fluorescence was selected to form the basis of the t-A07 strand.

The cells was maintained in a monolayer culture with RPMI 1640 (25mM Hepesbuffer and L-Glutamine) with Fetal Bovine serum and Penicillin (10.000 units/ml)- Streptomycine (10000 ug/ml) as the medium.

The cells were transformed into spheroids by transfering 1 x 10^6 t-A07 cells to 30 ml medium and genecitin (700mg/L). The bottles vere flushed with 5% CO_2 in air, and tilted at 37°C the next 36 hours, while the cells aggregated into spheroids. The cultures were then transferred to an incubator keeping 37°C, where they remained until the spheroids had grown to the desired size (2-3 weeks)

Animal modell

Adult female BALB/c-nu/nu mice, weighing between 22-28 g, were used as host animals for the xenografted tumors. The mice were bred at the animal department of our institute, and were kept under spesific pathogen free conditions. They were kept at constant temperature (37.0 +/- 0.5 °C) and humidity (50-60%). Sterilized food and sterilized water were given ad libitum. The mice vere treated in accordance with the Helsinki rules.

Anesthesia
Equitesine® (Phentobarbithale) (0.04ml/10g) was used as anesthesia before surgery and observations. Temgesic® (Buprenorfin) (0.1 ml) was given as analgetika during surgery. Fucidine® crème (Fucidic acid) was applied as antibiotics on the stitches after surgery, and again at any sign of inflammation.

Experimental protocol:
The dorsal skin fold chamber
The dorsal skin fold chamber was implanted by folding the loose dorsal skin on the mouse, forming a double skinfold. Using the two major arteries in the dorsal skin fold as markers of location, a circular incision was made, removing all the layers of skin and facia on one of the sides. Directly opposite the skin was split between the epidermal and dermal layer, taking great care not to harm the vessels, and then the epidermis was removed. This resulted in a thin curtain of dermal and subcutal tissue. A framework made of PEEK was incerted from both sides, sandwiching the tissue. Into the framework we fixed two transparent plastic disks, using titanium clips, so that the tissue was freely visible from both sides. The distance between the windows was 250µm . The total weight of the chamber was approximately 1,55g. The implanted chamber did not seem to hinder the mice in any way. This procedure is explained more fully in and upcoming article from our institute (by Graff BA).

Size of the spheroids implanted varied, the number of cells being between 10^4 and 5x 10^4. Using a 100 µl Hamilton-syringe, the spheroid was gently placed in the middle of the chamber on the facial side of the tissue. The plastic disc was then replaced on top. To ensure that the spheroid was placed centrally in the chamber, the operation was done underneat a stereo lens emitting fluorecent light so that the GFP-transfictated spheroid was clearly visible. The spheroid would then grow inside the chamber, and the vascular network was monitored.

Imaging
The anesthezised mouse was placed in a special holder, fixing the chamber underneath a light microscope. The tumor vasculature hierarchy was assessed using a first pass imaging technique, in witch a fluorescent contrast was injected i.v. into the tail vein, while filming the vascular network of the tumor. The contrasts used were FITC-dextran (fluorescein isothiocyanat labeled dextran), Texas red- dextran or TRITC-dextran (Thetramethyl rhodamin dextran). The bolus of contrast fluid was followed, giving a visual picture of how contrast spreads in the vascular network (fig.1).

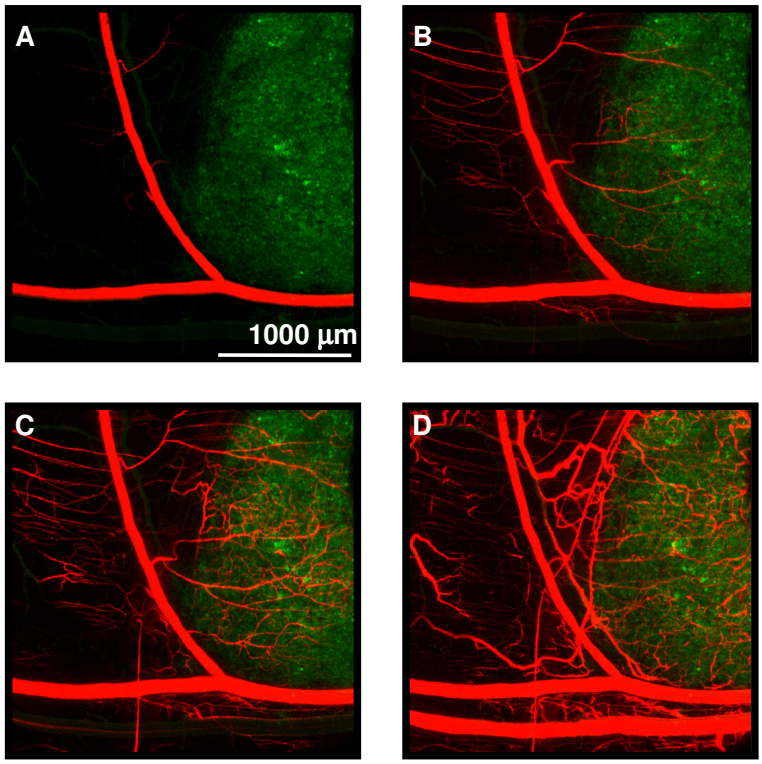


Fig. 1

Fig 1 A-D:
The 4 frames are taken from a first pass imaging sequence, and show the blood supply to tumour tissue and normal tissue during an i.v. contrast bolus injection. Green indicates tumour tissue, black indicates normal tissue and red indicates the contrast filled vessels.
A: The datum point, where the contrast bolus has only just reached the main arteries. B: 0,66 seconds: the bolus has reached the tumour supply vessels and arterioles . C: 1,33 seconds: the contrast has filled most of the tumor vessels, and normal tissue capillaries. D:4,67 seconds: the contrast fluid drains from both the normal tissue and the tumor tissue into the venules

The recording was primary taken from the tumor surface, but ocationally supplemened by recordings from the dermal side of the tumor. To keep the temperature in the skin constant during observations, we used a hot air generator (*37°C*).
The images were recordered using a Hamamatsu digital black and white camera (C4742-95) and an Olympus U-CMA03 microscope, and the computer programs *Aquacosmos© and Wasabi© from Hamamatsu Photonics.*
7 mice with chambers were used and a total of 24 first pass imaging films. Still shots of the tumor, both with compound light and fluorecent light, was always taken in the exact same frame as the first pass imaging film.

Measurements and analyses

Time to peak (TTP) was used as a measurement to compare the blood flow through different vessels. The injected contrast reaches a maximum of inensity when it fills the vessel, which is denoted the peak. Measurement windows were drawn on different kinds of vessels, and the intensity in the windows were measured (Fig 3A) To make the films comparable, the peak of the artery is denoted the zero point. The time from the peak of the artery to the peak of the other types of vessel is denoted the time to peak (TTP) (fig.3 B), and then analyzed (fig. 3 C) Fig .3 D-F shows the validity for the use of TTP as a measure of blood flow, and first pass imaging.

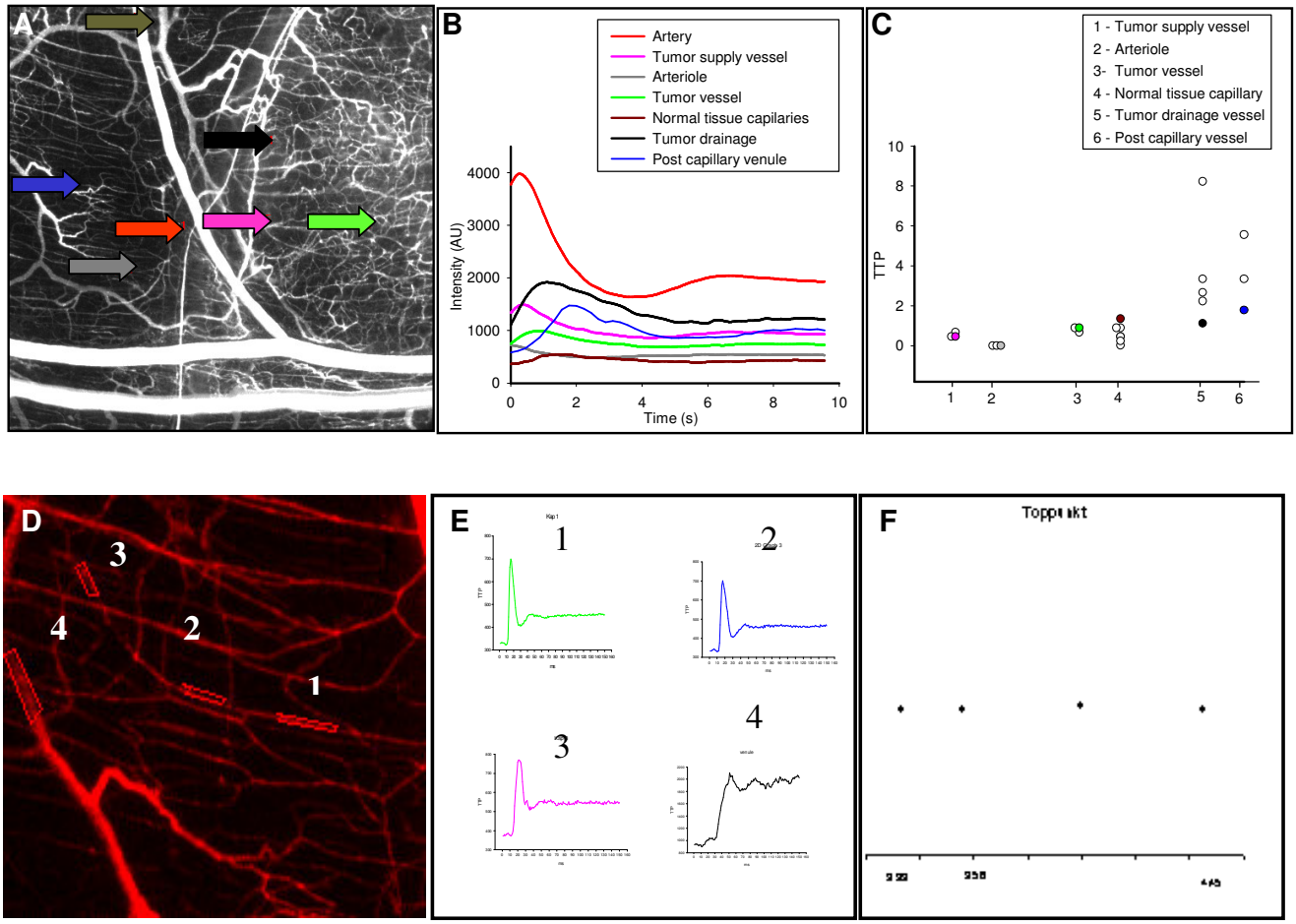


Fig. 2

Fig2 A-C: Illustrates the technique used to analyse the recordings
A: Measurement windows are drawn inside different types of wessels. B: these graphs show how the light intensity fluctuates as the bolus of contrast moves through the measurement window. The time of the peak is measured (TTP). C: TTP for the different types of vessels.
D-F illustrates that we can follow a boulus of contrast in a single vessle, and thus determine the direction of flow. For comparison the vessel it drains into is also measured.
D: Measurement windows drawn in one vessle, and its appurtenant drainage vessel.

E: A graphical view of the light intesity in different parts of the vessel, as the boulus of contrast passe through F: The TTP increases as the bolus moves along the vessle and into the TDV (TTP in seconds is the parameter on the x-graph).

Statistical analysis

To check for significant difference between TTP for different vessels, the Man Withney (Wilcoxon-White) test was used.

Definitions

Arterioles: vessels supplied by an artery, not leading a tumor vessel
Tumor supply vessels (TSV): vessels supplied by an artery, leading to a tumor vessel
Normal tissue cappilaries (NTC): small, parallel vessels outside the tumor, originating from arterioles, and draining into the venules.
Tumor vessels (TV): small vessels, inside the tumor tissue, supplied by TSV.
Post cappilary venules (PCV): vessels the NTC drains into.
Tumor drainage vessel (TDV): small vessels TV drains into, located inside or very near the tumor.

RESULTS

The spheroids became vascularized 4-7 days after implantation. The primary vasculature was chaotic and heterogeneous in vessel diameter and vascular density. After ~7 days, the vasculature had a characteristic regular pattern (fig 3)

The tumors were supplied with blood from normal, pre-existing arterioles and the drainage was through pre-existing venules, which both were connected to the developing tumor vasculature (fig 4)

The arteries showed no signs of change, but the venules was observed to undergo significant morphological changes as their proximity to the tumour increased. This will be presented in another publication from our group (by Murata R)

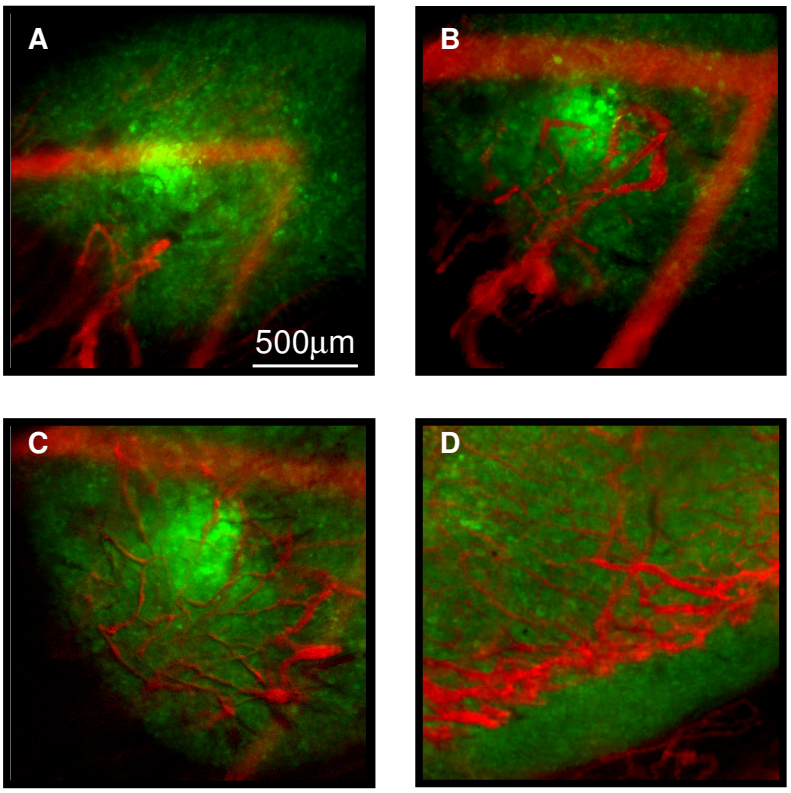


Fig. 3

Fig 3, A-D This picture sequence describe the different pattern of vessel development in a tumor over a time period of 7 days. Green indicating tumor cells, red indicating contrast filled vessels. A: The first functional blood wessels in the tumor, 7days after implantation. B: The vascular network 8 days later. Note the loopy and heterogenous apperance of the vessels. C: 9 days after implantation. The vessels are still heterogenous in regard to vessle diameter, but the network is less chaotic D: 13 days after implantation. The tumor vessle network located centrally in the tumor have a organized "fishnet" structure, with many interconnecting branches. The tumor vessels drain into the periphery, where a morphologicaly distinct wessle structure has appeared. This structure encompass the tumor, and appeared in most of the chambers that was allowed to grow to this size. Note that tumor tissue extends beyond this pattern. This wessle pattern is what we denote "a mature tumor network".

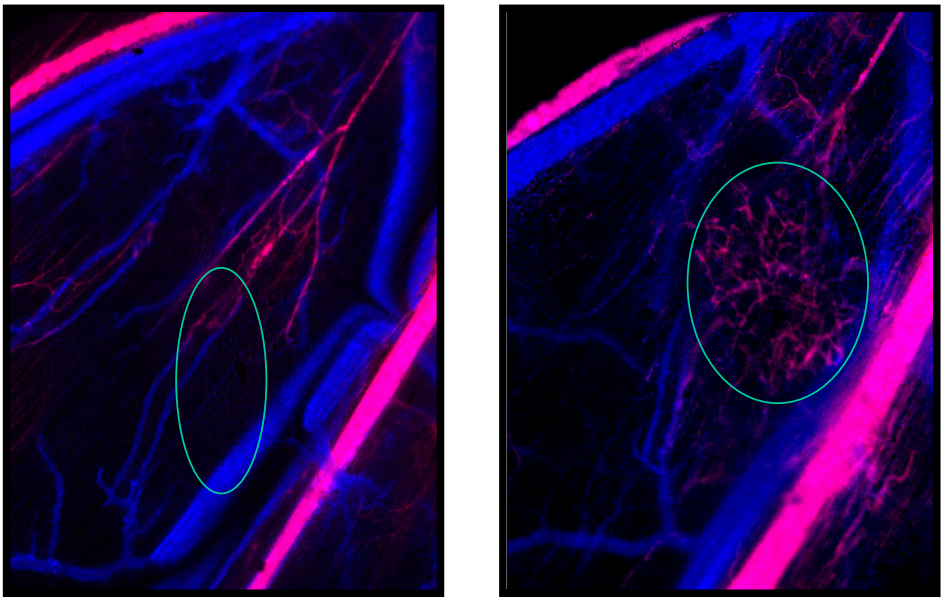
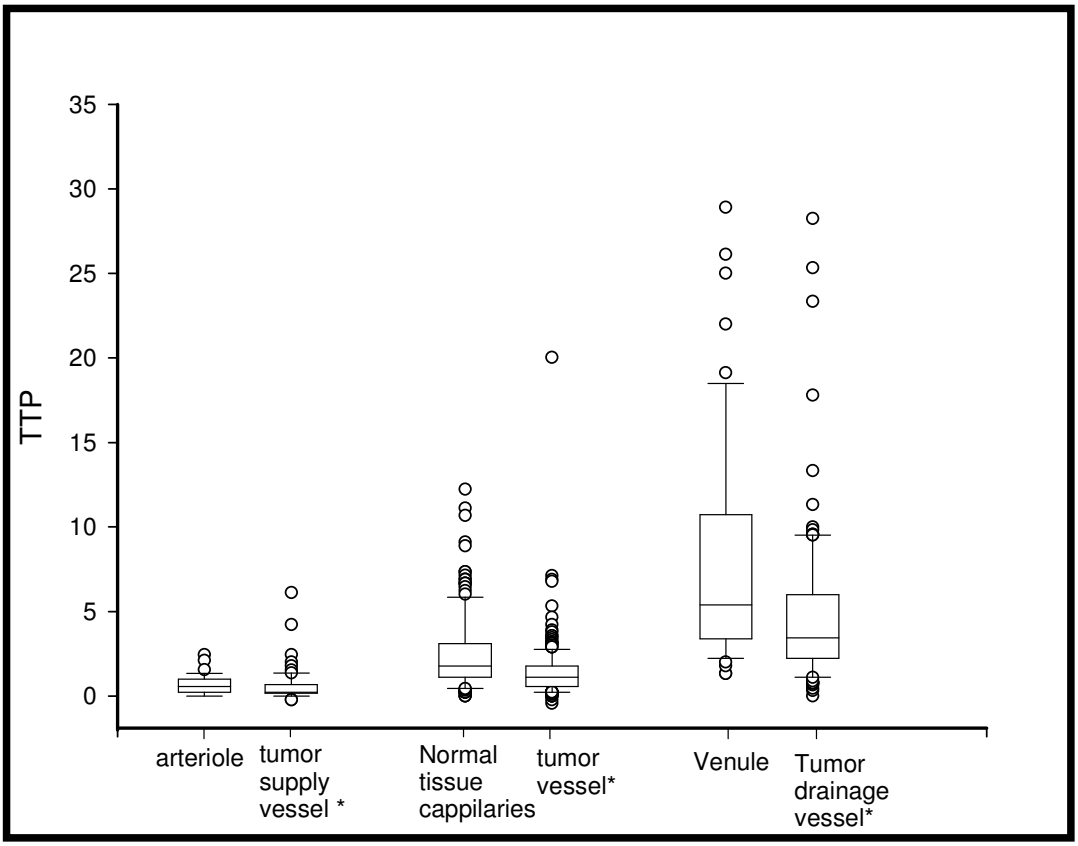


Fig.4

Fig 4 A+B: The pictures are from two different first pass imaging sequences, four days apart. They are made by picking two frames from each sequence, one in the beginning (pink) where the contrast bolus has reached only the supply vessels, and one in the end (blue) where the contrast bolus is draining into the drainage vessels. The location of the tumor is marked by the green ring A: Shows the vessels in the normal tissue the day after implantation of the tumor. B: The same location 5 days after implantation, when the tumor has established a functional network. The pink vessle now supplies the tumour network (also pink), and the blue vessle drains it. Note that the supplying and draining vessle is still the same, this shows how the tumor network is connected to the existing vessels.

TTP for different types of blood vessels is presented in Fig. 5. There was a significant difference in TTP being higher for NTA compared to TS ($p = 0.0183$). The same is registered for NTC having a higher TTP than TV ($p = 0.0017$) and for PCV having a higher TTP than TD ($p = 0.0125$). TS has a significantley lower TTP compared to TV (P=<0,0000000001).

A great heterogeneity in perfusion of NTC could be observed, both prior to and after the implantation of the spheroid and appearance of the tumor vessel network.



* Vessle in tumour tissue

Fig. 5

Fig.5 Box plot showing the TTP measured for the normal vessels and their tumor equivalents. Note that the vessels located inside the tumor, supplying or draining it, have a significantly lower TTP than the normal tissue vessels.

It was observed that in the tumours with a mature vessel network, the contrast bolus spread through the tumor with a heterogeneous time lag. (fig 6)

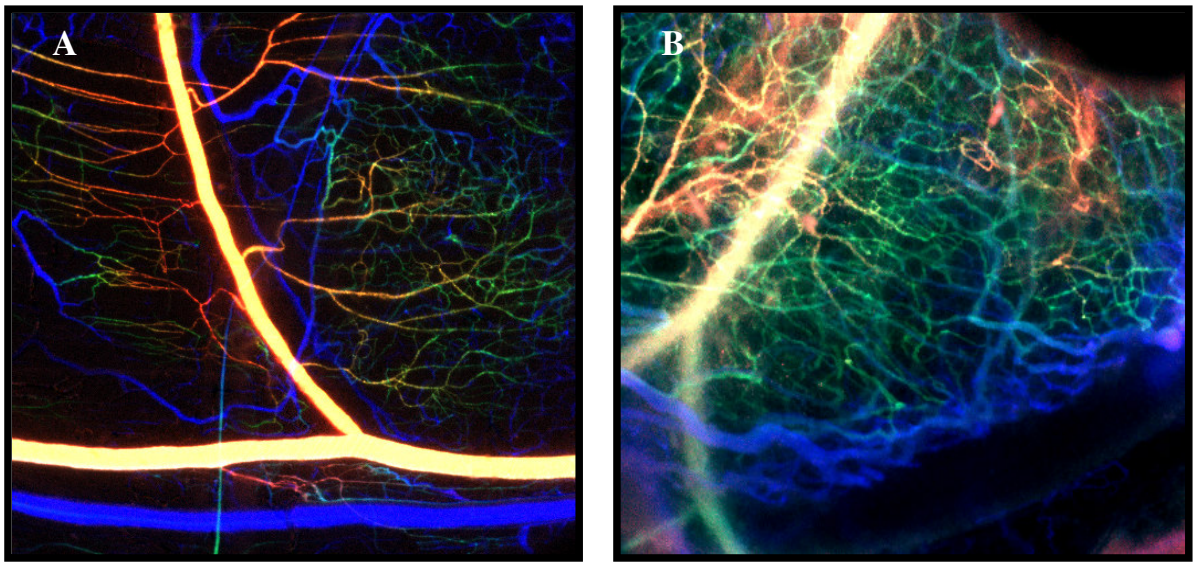


Fig 6

Fig 6 These two frames is each a collage of three pictures from the same recording session. They are colored so that the red-yellow parts of the network is where the bolus of contrast appears first (A:0,34 sec, B:0 sec), the green parts is where it flows next (A:1,33 sec, B:1,33 sec), end the blue part is where it drains to in the end (A: 4,67 sec, B: 5,34 sec). Note the heterogenity of color inside the tumor vasculature, with red and blue "islets" in between the green.
A: To the right we have the tumor vessel network (aproximatley half of the tumor is shown, with the tumor center beeing near the right side of the picture), and on the left side of the artery, we have normal tissue. Note the smooth and eaven change from red via green to blue in the normal tissue vasculature as compared to the heterogenous change in the tumour vasculature. B:The center of the tumor is towards the upper left corner, and the avascular perifery of the tumor can be seen in the lower right corner. Note the blue drainage area in the perifery.

DISCUSSION

I.v contrast combined with still-pictures can show how a tumor (and normal tissue) is vascularizised, and the development of the vasculature. First pass imaging is a unique way to broaden the usefulness of contrast, so that it can visualise the dynamic situation, and second to second changes in the in vivo tumor vasculature.
TTP for the TSV is significantly lower than both the NTA and the TV, showing that the blood supply to the malignant melanoma xenographt must be arterial in origin. In addition, this is seen directly in the first pass imaging recordings, as illustrated earlier (fig.1) Using the first pass imaging technique we discovered that normal tissue vessels reaches the point of maximum intensity later than the similar tumor vessels. The implication is thus that the blood passes faster through the tumor than the normal tissue.
Also there was a greater heterogeneity (significant difference) in when the capillaries reaches TTP compared to the tumor vessels, this could be because normal regulatory mechanisms is disrupted in tumor tissue.

The tumour is supplied from pre-existing arterioles, and there is little or no morphological change of these vessels as the tumor vascular network matures. The tumor is drained into pre-

exsisting venules to begin with, but as the tumor encompass these vessels, they undergo morphological changes, and seem to disappear. A disorganised periferal draining system appears shortly after, draining into the veins. The draining system consists of vessels that are morfologicaly (especially in diameter) different from central tumour vessels. The development of the tumour vasculature and drainage system is studied closer in an upcuming article from our group (by Murata R).

In the tumors with a mature vessel network, the i.v. contrast bolus did not spread evenly throughout the tumor, but rather reached some parts early and some parts later. This did not occur in the same degree in tumours with a newly developed and caotic vessel network. In addition, the intratumoural variation was smaller than the intertumoral, and as such it is not visible on the composite graph (fig.5) Our conclusion to this is that there is a heterogeneity in the length of the pathway the blood has traveled before it reaches the different regions of the tumor. This might lead to a heterogeneous oxygenation of the tumor. Witch clinical implications a heterogeneous oxygenation will have is not possible to determine from this study, as we do not know if the heterogeneity in perfusion is great enough to make some areas hypoxic, or what conditions that would be required to do so. As mentioned by Streit *et al* 2003, subpopulations of malignant melanoma cells has been shown to survive under conditions of hypoxia and metabolic stress, and melanoma xenografts has been shown to be heterogeneous with respect to vascular dependency and hypoxia toleration.

To compare results we had to have a standarisied zero point. The TTP for the main artery seen in the window chamber is used for this. It cannot always be visualized if all the arterioles, TSV, capillaries and tumor vessels originate from this artery. The time lag between different arteries is so small that we usually cannot measure it using the TTP metode, so we assume that the impact from this error is negligible.
Measuring the TTP, it should be taken care that there are no other vessels crossing the vessel that are measured, as this might disturb the value of the peak time and distrupt the light intensity curve to some extent. Usually it is possible to determine the TTP for each of the vessels, and if not the measurement window can be moved away from the intersection. A great vessel close to a smaller vessel may alter the measured TTP of the smaller vessles, by illuming the ajacent area. By checking that the measured TTP corresponds to the visual, this problem is eliminated.

Oxygenation of a tissue is dependent on the blood perfusion. Using the first pass imaging technique it is possible to study the blood perfusion of a tissue in vivo, and also see how it develops and react to different stimulation. A pubmed search showed that the first pass imaging technique has not been used in this way before.

The next logical step is to study how medications will influence the TTP in tumors, thereby assessing their impact on blood perfusion, development and maturation of the vascular network.

We thank Bjørn Anton Graff and Einar K Rofstad for their assistance.

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